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Proteinuria, lipoproteins and renal apolipoprotein deposits in uninephrectomized female analbuminemic rats

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Proteinuria, lipoproteins and renal apolipoprotein deposits in uninephrectomized female analbuminemic rats. To elucidate the pathogenetic role of hyperlipidemia *per se* in the development of glomerulosclerosis, severely hyperlipidemic female analbuminemic rats (NAR) and mildly hyperlipidemic male NAR were studied for a period of 37 weeks after uninephrectomy (UNX). Plasma cholesterol increased from 6.3 ± 0.4 (week 4) to 11.9 ± 0.6 mmol/liter (week 37) in the ♀ NAR, and from 4.3 ± 0.1 to 6.4 ± 0.5 mmol/liter in the ♂ NAR in the same period. Plasma protein concentration was also consistently higher in ♀ NAR (60 ± 1 g/liter) as compared to ♂ NAR (52 ± 1 g/liter). Plasma viscosity was higher in ♀ NAR than in ♂ NAR, but there were no differences in blood viscosity. Proteinuria increased progressively in the UNX ♀ NAR from 25 weeks after surgery, reaching a final value of 141 ± 37 mg/day. No proteinuria occurred in the UNX ♂ NAR (final value 15 ± 2 mg/day). Glomerular capillary pressure, measured prior to the onset of proteinuria, was not significantly different in UNX ♀ NAR and UNX ♂ NAR. At the end of the study glomerulosclerosis and lipid deposition was only found in the UNX ♀ NAR. Throughout the study hyperfiltration and hyperperfusion, relative to the one-kidney clearances of the sham-operated (2K) animals, were not different in UNX ♂ and ♀ NAR. No differences were observed in blood pressure. Hypertrophy, evaluated by glomerular diameters, was less pronounced in UNX ♀ NAR (174 ± 3 μm) than in UNX ♂ NAR (190 ± 7 μm). Glomerular diameters in 2K ♀ and ♂ NAR were similar (respectively 158 ± 2 and 157 ± 4 μm). Plasma apo B levels were similar (2K ♀ NAR: 204 ± 8 U; 2K ♂ NAR 204 ± 13 U), but cholesterol and triglyceride content of apo B-containing lipoproteins, namely VLDL, IDL and LDL, was increased twofold in the ♀ NAR as compared to the ♂ NAR, implying a larger particle size in the ♀ NAR. Deposition of apo B and apo E was observed in the glomerular mesangium of UNX ♀ NAR, particularly in sclerotic lesions. Glomerular apo A-I deposits were localized primarily in visceral epithelial cells and were not associated with sclerotic lesions. The development of proteinuria and glomerulosclerosis after UNX in ♀ NAR but not in ♂ NAR may depend upon differences in plasma lipoprotein composition, but is apparently not related to differences in whole kidney hyperfiltration and hyperperfusion, glomerular capillary pressure, or blood viscosity.

A variety of pathogenic mechanisms contribute to the progression of renal disease leading to glomerulosclerosis. Besides a rise in glomerular capillary pressure (P_{GC}), metabolic disturbances, notably hyperlipidemia, may play a role [1]. In a 5/6 nephrectomy model of chronic renal failure, lipid-lowering agents considerably delayed the development of glomerulosclerosis [2]. In uninephrectomized rats a high dietary fat content induced glomerulosclerosis [3]. In a previous study in 3/4 nephrectomized Wistar rats we found that besides proteinuria, hyalinosis, and endocapillary proliferation, hypercholesterolemia also correlated with the development of glomerulosclerosis [4].

Male rats are used in most studies of renal ablation because of the rapid development of proteinuria and glomerulosclerosis [2, 4–6]. Female rats are relatively resistant to the development of proteinuria after uninephrectomy [7] or subtotal nephrectomy [8]. The reason for this gender-linked difference, which seems to depend on testosterone [9], is probably the milder increase in P_{GC} and less pronounced glomerular hypertrophy in female rats [7, 9]. There were no significant differences in plasma cholesterol in uninephrectomized male and female rats [9]. From these observations one might conclude that the rise in P_{GC} and glomerular hypertrophy are the primary causes of the progression of renal failure. Metabolic disturbances may play a role in the presence of permanent glomerular hypertension only, for which systemic hypertension may be a prerequisite [10, 11]. In agreement with the above, it has been reported that aging male Nagase analbuminemic rats (NAR), which are characterized by mild hyperlipidemia [12], normal arterial pressure [12, 13], and low P_{GC} [13], develop no glomerulosclerosis [12, 13]. In contrast, we recently observed that normotensive female NAR, which are more severely hyperlipidemic than male NAR [12], do develop overt proteinuria by one year of age, suggesting an important role for the metabolic disturbance *per se* in the development of glomerulosclerosis.

In this study we tried to dissociate compensatory hyperfiltration and glomerular hypertrophy from the development of proteinuria to elucidate the pathogenetic role of hyperlipidemia *per se* in the development of glomerulosclerosis after uninephrectomy. To this end we monitored plasma lipids, renal function and proteinuria in male and female NAR repeatedly during a period of 37 weeks after uninephrectomy or sham operation, and measured glomerular diameter and sclerosis at the end of this period. Furthermore, to elucidate the role of plasma lipoprotein composition in the development of glomerulosclerosis, we also evaluated plasma apolipoproteins and lipoprotein composition in relation to glomerular apolipoprotein and lipid deposition.

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Renal damage has been found in rats subjected dietary hypercholesterolemia for 19 to 23 weeks [3, 14]. The damage tended to be more severe in rats that were also subjected to uninephrectomy [3, 14]. Dietary hypercholesterolemia increased P_{GC} to similar values in UNX and 2K rats four to six weeks after surgery, at which point the rats were virtually free of glomerular injury [14]. It is plausible that the difference in cholesterol and triglyceride levels in female and male NAR could be accompanied by differences in P_{GC}. Thus, we measured P_{GC} in uninephrectomized rats at a stage of impending proteinuria. In these rats we also measured blood and plasma viscosity in view of the association between blood viscosity, P_{GC} and glomerular injury [15] on the one hand, and the suggested relation with hyperlipidemia [14] on the other hand.

Methods

Animals

Male (♂) and female (♀) NAR (6 to 12 weeks old) from our own pathogen-free colony (which was founded with animals generously donated by Dr. S. Nagase, Tokyo, Japan) underwent right nephrectomy (UNX) or sham operation (2K) under fentanyl/diazepam anesthesia and sterile surgery. Body weight of the UNX and 2K groups was similar at the time of surgery: UNX ♂ 234 ± 12 g ($N = 8$), 2K ♂ 228 ± 11 ($N = 8$); UNX ♀ 187 ± 6 g ($N = 10$), 2K ♀ 188 ± 7 g ($N = 8$). The rats were housed behind barriers in filter-top cages (♂ 2 to a cage, ♀ 3 to a cage). Sentinel animals that were monitored regularly for infection by nematodes and pathogenic bacteria, as well as antibodies to a large number of rodent viral pathogens (ICLAS, Nijmegen, The Netherlands), were consistently negative throughout the course of the experiment. The rats were provided with a semisynthetic diet (Hope Farms, Woerden, The Netherlands) containing 20% casein-protein, 10% corn starch, 5% cellulose, 53% glucose monohydrate, and 5% soybean oil (by weight). The remaining 7% consisted of salts, vitamins, and trace elements. Food intake was measured continuously for the first seven weeks after surgery. Separate groups of UNX ♀ ($N = 6$) and UNX ♂ rats ($N = 6$) were studied 14 weeks after UNX. The protocol was approved by the Utrecht University Board for study in experimental animals.

Renal function

Urine was collected after 1, 4, 15, 25 and 32 weeks in the 37 week study and after 11 weeks in the 14 week study for the determination of creatinine clearance and urinary protein. The rats were placed in macrolon metabolism cages, with free access to food and water. Two consecutive 24-hour urine samples were collected. The animals were then anesthetized with Na pentobarbital (60 mg/kg), weighed and 1 ml of blood was sampled by puncture of the tail artery with a needle (25G). Blood was collected in heparinized tubes. Renal function was also determined in pentobarbital anesthetized animals by one hour ^{51}Cr-EDTA and ^{125}I-hippuran clearances after 4, 8, 19 and 33 weeks [16]. Tail cuff pressure was measured in conscious animals on the day before the renal function measurements (IITC, San Diego, CA, USA).

Micropuncture protocol

Micropuncture was performed 14 weeks following UNX. The rats were anesthetized with pentobarbital sodium (50 mg/kg i.p.) and prepared for micropuncture as described previously [17]. Briefly, following tracheal intubation, two PE-50 catheters were placed into the left jugular vein, one for infusion of solutions and the other for additional anesthetic. A catheter was placed in the left femoral artery to monitor mean arterial pressure. The left kidney was approached by a flank incision and placed in a Lucite holder with an agar wall around the kidney to form a saline well. A PE-10 catheter was placed in the left ureter for urine collections. Following surgery, a 60-minute equilibration period was observed. All animals were infused throughout the experiment with 0.9% NaCl containing 15% polyfructosan (Inutest, Laevosan, Linz, Austria) and 0.5% paraaminohippurate at a rate of 20 μl/min. The protocol consisted of two 40-minute periods, during which timed urine collections were obtained. Plasma samples were obtained at the beginning and end of the protocol. Two values for both stop-flow pressure and proximal tubular free-flow pressure were obtained in each rat, as described previously, using a servo-null pressure device (Instruments for Physiology and Medicine, San Diego, CA, USA). At the end of the protocol renal vein blood was sampled to measure PAH extraction.

Tissue processing

At the end of the experiment all rats were exsanguinated in the fed state by puncture of the abdominal aorta under pentobarbital anesthesia. Subsequently the left kidney was perfused in situ for one minute at a pressure of 100 mm Hg with heparinized (5 IU/ml) phosphate buffered saline, pH 7.4 (PBS), containing 6% sucrose. After sampling the cranial apex for lipid histochemistry, the remaining tissue was perfused for an additional two minutes with 0.025% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer. The fixative was removed by one minute of reperfusion with the PBS sucrose solution. The sample for lipid histochemistry was quickly frozen in liquid nitrogen. Coronal tissue slices, 1 mm thick, were cut from the midportion of the kidney, fixed in 2% paraformaldehyde in PBS at 4°C for three hours and processed for glycol methacrylate embedding as described previously [18].

Staining procedures for light microscopy

For the immunohistochemical localization of apolipoproteins and macrophages plastic sections were processed as described previously [18]. In short, sections were incubated in appropriate dilutions of rabbit anti-rat polyclonal antibodies directed to apo A-I, apo E [19], apo B [20] and mouse anti-rat macrophage antibody (ED1, provided by Dr. C. Dijkstra, Free University, Amsterdam, The Netherlands). Endogenous peroxidase was blocked in PBS, containing 0.06% H_2O_2. Second step antibodies, peroxidase-conjugated swine anti-rabbit IgG (Dakopatts, Glostrup, Denmark) were applied for one hour at room temperature in a dilution of 1 to 20 in PBS, containing 5% normal rat serum. Counterstaining was performed using periodic acid-Schiff and Mayers hematoxylin. To improve morphological detail, sections were covered with two drops of complete Technovit 8100 medium (solution A+B, 30:1) as used for embedding.

Control experiments were performed to test the specificity of the antibodies. Previously, double immunodiffusion against purified antigen ruled out reaction of the polyclonal anti-apolipoprotein A-I, E [19], and B (van Tol, unpublished observations) sera with unrelated antigens in plasma. Rabbit anti-rat antibodies to apo A-I, apo B and apo E were absorbed with normal rat serum

(containing all apolipoproteins) in a concentration of 5% for 15 to 30 minutes at room temperature. Subsequently, staining results of the absorbed antibodies were compared with the unabsorbed. Absorbance completely removed specific glomerular immune reactivity for apolipoproteins. Routine control experiments, that is, replacement of the first antibody by non-immune rabbit serum or PBS, were consistently negative. Sections were examined under the light microscope to determine the exact localization of apolipoproteins and the number of glomerular macrophages.

Lipid histochemistry

Glomerular and tubular lipid deposits were scored in 4 μm frozen sections stained by the Oil Red O method (ORO). Glomeruli were scored semiquantitatively on a scale of 0 to 4+ [18]. Briefly, if 25% of the glomerulus was affected, a score of 1+ was adjudged, 50% was scored as 2+, 75% as 3+, and 100% as 4+. The ultimate score was then obtained by multiplying the degree of change by the percentage of glomeruli with the same degree of injury and additions of these scores. Thus the maximum score for glomerular lipid deposits was 400. A total number of 50 glomeruli per animal was scored moving from cortex to medulla. Tubules were also scored semiquantitatively on a scale of 0 to 4+. Focal deposits in either cortex or medulla were scored as 1+, focal deposits in cortex as well as medulla were scored as 2+, diffuse light deposits in cortex and medulla 3+, and diffuse severe deposits in cortex as well as medulla 4+. Thus the maximum score for tubular lipid deposition was 4.

Glomerular diameter

Glomerular diameter was measured in PAS stained sections as described previously [4]. At least 50 glomerular profiles (Bowman's capsule) per kidney were traced. Mean glomerular diameters were calculated according to van Damme and Koudstaal [21].

Focal glomerulosclerosis

Focal glomerulosclerosis (FGS) lesions were defined by the presence of focal and segmental glomerular scarring and obliteration of glomerular capillaries with increased mesangial cellularity, mesangial matrix expansion and adhesion formation between the tuft and Bowman's capsule. The severity of FGS was scored semiquantitatively on a scale of 0 to 4+ [22] in PAS stained sections. Fifty glomeruli were scored per animal with the same system that was used to assess lipid deposition. Thus the maximum score for FGS lesions was 400.

Biochemical analyses

Plasma and urinary protein was determined by the Bradford method. Plasma and urinary creatinine was determined colorimetrically (Sigma). Urine and plasma inulin concentrations were assessed photometrically with indoleacetic acid after hydrolyzation to fructose. Urine and plasma PAH concentrations were assessed photometrically by a chromogenic aldehyde reaction. Plasma colloid osmotic pressure was measured using a strain gauge micro-oncometer. Enzymatic methods were used for the determination of total plasma cholesterol and triglycerides. The kits were obtained from Boehringer GmbH (Mannheim, Germany). Plasma concentrations of apo A-I, and apo E were measured by electroimmunoassay as described previously [19]. Plasma apo B was determined by radial immunodiffusion, using a specific antiserum raised in rabbits against purified rat LDL [20]. Plasma apo B concentrations, expressed in arbitrary units (A.U.) because of the insolubility of purified apo B and the lack of sufficient quantities of pure apo B for use as an absolute standard, were calculated as percentages of a rat serum standard pool (obtained from 50 rats) run simultaneously on the plates with the plasma samples. Six different dilutions of the serum standard pool were run on each plate. All samples were run in triplicate. Fibrinogen was measured by electroimmunoassay. Rat fibrinogen was purchased from Sigma (St. Louis, MO, USA) and goat anti-rat fibrinogen from Nordic (Tilburg, the Netherlands). Three different dilutions of the standard were run on each plate. All samples were run in duplicate. Viscosity measurements of reconstituted blood (hematocrit 42%) and plasma were performed on a Contraves Low Shear 30 viscosimeter (Contraves A.G., Zurich, Switzerland) at 39°C at 7 shear rates (6-50 s^{-1}).

Lipoprotein isolation by density gradient ultracentrifugation

Plasma lipoproteins were separated in four UNX ♀, four 2K ♀, four UNX ♂ and four 2K ♂ by density gradient ultracentrifugation [20] into seven fractions (chylomicrons and VLDL, d < 1.006 g/ml; intermediate density lipoprotein, IDL, d 1.006 to 1.019 g/ml; low density lipoprotein; LDL1, d 1.019 to 1.04 g/ml; LDL2, 1.04 to 1.063; HDL2, d 1.063 to 1.125 g/ml; HDL3, d 1.125 to 1.21 g/ml and an infranatant with a d > 1.21 g/ml). The subdivision of LDL into LDL1 and LDL2 was performed to separate the apo B containing lipoproteins from the other particles present in the total LDL density range of 1.019 to 1.063 g/ml [20]. Lipoprotein TC and TG were measured as described above.

Urinary protein composition

Protein composition of 16 urine samples was analyzed by polyacrylamide gelelectrophoresis in the presence of sodium dodecyl sulphate, as described previously and evaluated with an image analysis system [20, 23]. The polyacrylamide gels consisted of a linear gradient of acrylamide from 4 to 10% and a constant 2.6% N′N′-bis-methylene-acrylamide. Standardization was performed using a mixture of two commercial preparations (Sigma 6H and Serva 5) containing proteins with molecular masses ranging from 6.5 to 205 kD. The molecular masses of the unknown proteins were estimated by using linear extrapolation between the two nearest standard proteins in the gel. Sequential urine samples were analyzed from 4 UNX ♀ rats at 4, 15, 25 and 32 weeks post-UNX.

Calculations and statistics

Clearances and excretions were calculated using standard formulae. GFR during the micropuncture experiment was assessed from inulin clearance, RPF from the clearance of PAH, after correction for the PAH extraction ratio. P_{GC} was estimated from

$$P_{GC} = SFP_0 + \pi_a$$

in which SFP_0 is the stop flow pressure during conditions of zero-flow and π_a, the colloid osmotic pressure in the afferent arteriole, which is presumed to equal systemic π. Glomerular transcapillary hydraulic pressure (ΔP) was estimated from

$$\Delta P = P_{GC} - P_T$$

in which P_T is the proximal tubular pressure under free-flow conditions.

Hyperfiltration was calculated by normalizing the creatinine or EDTA clearance found in the UNX animals to 50% of the mean value found in the 2K animals at the same time point post-uninephrectomy. Hyperperfusion was similarly calculated using the hippuran clearance. Proteinuria, glomerular injury score and glomerular lipid deposition were log-normalized as the distribution was skewed. Differences in blood viscosity were determined by analysis of covariance after log-log transformation of the data. Values were subjected to a two-way completely randomized analysis of variance. If a variance ratio (F) reached statistical significance ($P < 0.05$), the differences between the means were analyzed by the least significant difference test [24], using the interaction mean square error to calculate the least significant difference. Results are expressed as arithmetic means with their standard errors (SEM), with the exception of lipoprotein composition ($N = 4$) where standard deviation (SD) is presented.

Results

Characterization of the model

No differences were observed in food intake or body weight during the course of the experiment between the UNX or 2K animals in either ♂ or ♀ rats. Final body weights of the UNX and 2K groups were similar at the time of sacrifice: UNX ♂ 563 ± 24 g ($N = 8$), 2K ♂ 593 ± 20 ($N = 7$); UNX ♀ 287 ± 6 g ($N = 10$), 2K ♀ 315 ± 15 g ($N = 7$). Two rats died in anesthesia, one 2K ♂ at 15 weeks, and one 2K ♀ at 33 weeks. Plasma cholesterol levels were consistently higher in the ♀ NAR as compared to the ♂ NAR ($P < 0.01$; Fig. 1). Only in the terminal blood samples (after 37 weeks) was cholesterol higher in the UNX ♀ NAR than in the 2K ♀ NAR ($P < 0.05$). Plasma triglyceride levels were higher in the ♀ NAR than in the ♂ NAR at 25 and 37 weeks ($P < 0.01$; Fig. 1), but were not increased by UNX. Plasma protein concentration was always higher ($P < 0.01$) in the ♀ NAR (60 ± 1 g/liter) than in the ♂ NAR (52 ± 1 g/liter), but was not affected by UNX (Fig. 2). Tail cuff pressure was approximately 150 mm Hg at 4, 8, 19 and 33 weeks in all four groups (Table 1).

Proteinuria

At all time points between 1 and 33 weeks after surgery, the highest individual loss of urinary protein in a ♂ rat (irrespective of whether there were 1 or 2 kidneys present) was 35 mg/day. During the first 15 weeks the highest loss of urinary protein in a ♀ rat was 16 mg/day. Mean values were lower in the first 15 weeks in the ♀ NAR than in the ♂ NAR ($P < 0.01$), and there was no effect of UNX (Fig. 3). However, at 25 weeks after surgery 30% (3 of 10) of the UNX ♀ rats demonstrated manifest proteinuria (45, 67 and 172 mg/day) as compared to 13% (1 of 8) of the 2K ♀ rats (42 mg/day). This increased to 80% (8 of 10) of the UNX ♀ rats at 32 weeks as compared to 29% (2 of 7) of the 2K ♀ rats. Mean urinary protein loss at 32 weeks was 141 ± 37 mg/day in the UNX ♀ rats versus 17 ± 7 mg/day in 2K ♀ rats ($P < 0.01$).

Electrophoresis of sequential urine samples of four UNX ♀ rats that became heavily proteinuric revealed considerable loss of glomerular protein selectivity (the results found in 2 of these animals are shown in Fig. 4). At 4, 15 and 25 (3 out of 4 rats) weeks, when urinary protein excretion was 7 ± 1 mg/day, three bands at 13.5, 20.0 and 29.5 kDa can clearly be identified. At 25 (1 out of 4 rats) and 32 weeks, when marked proteinuria (230 ± 39 mg/day) was present, additional bands became prominently visible between 50 and 75 kDa. In all four animals the bands at 13.5 and 20.0 kDa that were prominent in the early stages could not be detected at this later stage, whereas a sharp band could be identified at 12.3 kDa. Note the absence of a dominant albumin band at 68 kDa in all samples.

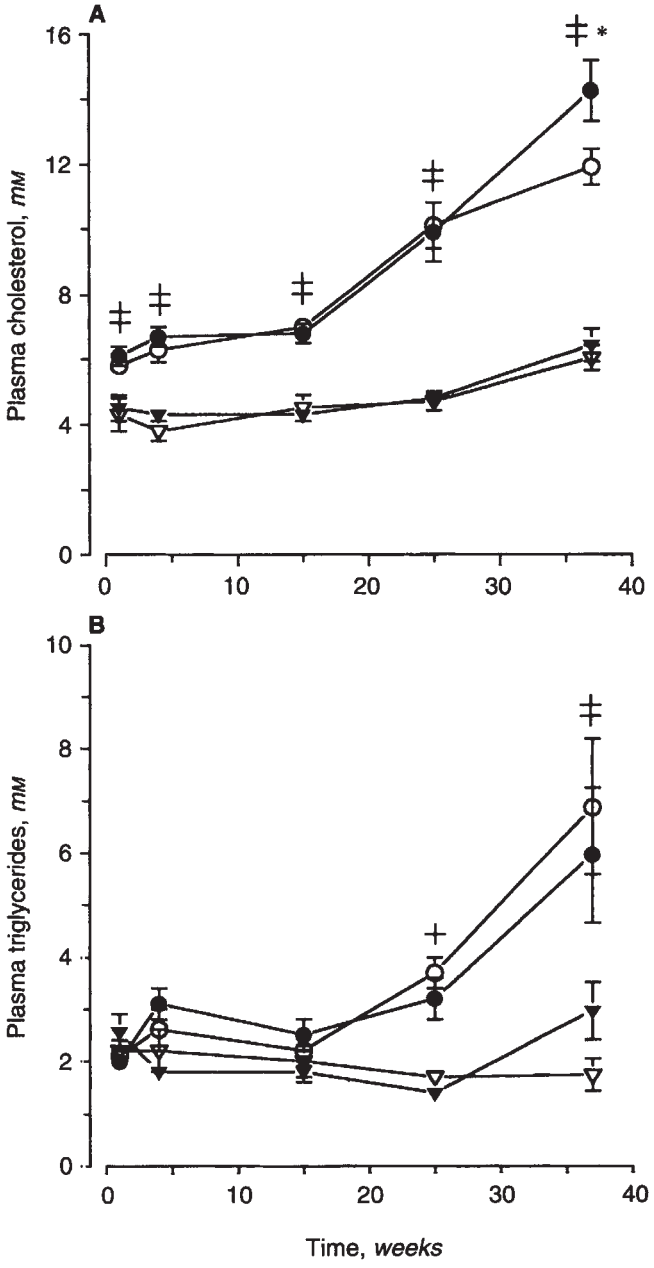

Fig. 1. *Plasma cholesterol and plasma triglycerides in uninephrectomized (UNX) and control (2K) female and male analbuminemic rats.* Symbols are: (○) female 2K; (●) female UNX; (▽) male 2K; (▼) male UNX. Data are mean ± SEM. Females vs. males †$P < 0.05$ ‡$P < 0.01$; UNX vs. 2K *$P < 0.05$.

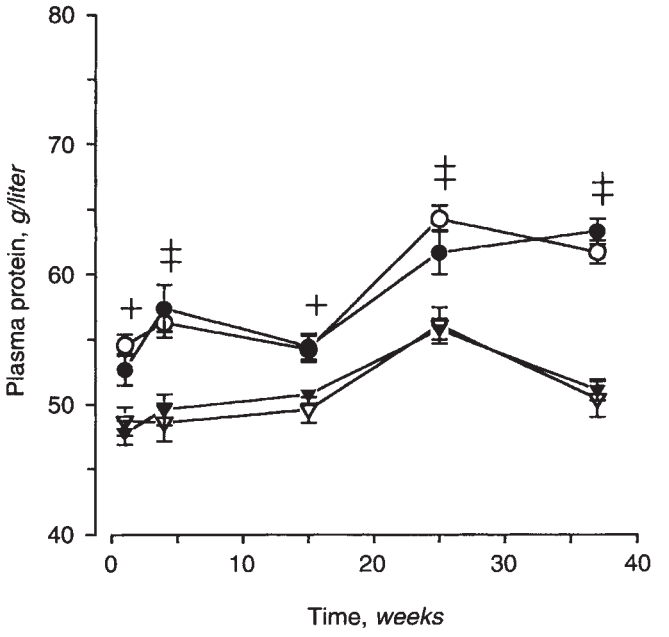

Fig. 2. *Plasma protein in uninephrectomized (UNX) and control (2K) female and male analbuminemic rats.* Symbols are: (○) female 2K; (●) female UNX; (▽) male 2K; (▼) male UNX. Females vs. males $+P < 0.05$, $+P < 0.01$.

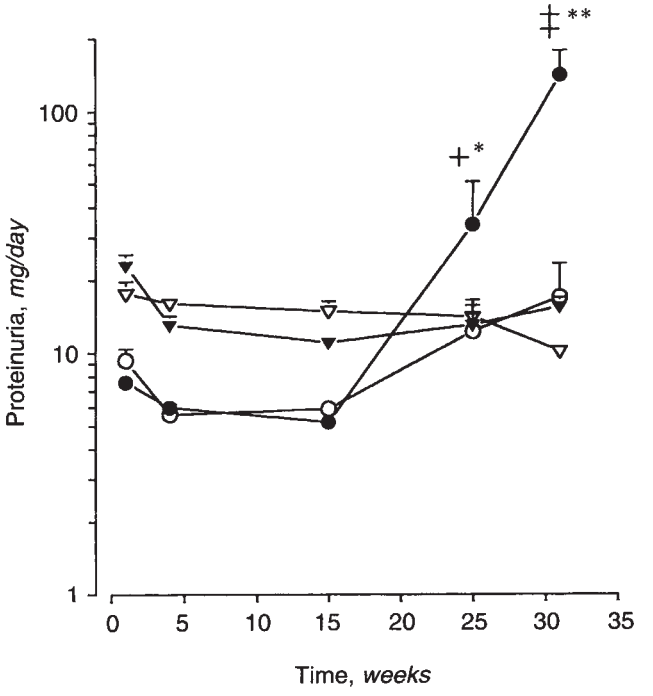

Fig. 3. *Proteinuria (log scale) in uninephrectomized (UNX) and control (2K) female and male analbuminemic rats.* Symbols are: (○) female 2K; (●) female UNX; (▽) male 2K; (▼) male UNX. Females vs. males $+P < 0.05$, $+P < 0.01$; UNX vs. 2K $*P < 0.05$, $**P < 0.01$.

Table 1. Tail-cuff pressure (mm Hg) in control (2K) and uninephrectomized (UNX) female (♀) and male (♂) analbuminemic rats 4, 8, 19 and 33 weeks after surgery

	2K ♀	UNX ♀	2K ♂	UNX ♀
N	8	10	7	8
Week 4	162 ± 5	146 ± 8	143 ± 8	165 ± 6
Week 8	137 ± 4	157 ± 5	171 ± 6	155 ± 5
Week 19	140 ± 4	150 ± 4	160 ± 7	157 ± 6
Week 33	149 ± 2	153 ± 3	155 ± 6	152 ± 5

Data are mean ± SEM.

Whole kidney renal hemodynamics

In UNX rats, hyperfiltration, as assessed by the 24-hour creatinine clearance repeatedly between 1 and 33 weeks after surgery, varied between 150 and 195% of the mean control 1-kidney filtration (Fig. 5). Hyperfiltration as assessed repeatedly by the one hour EDTA clearance varied between 140 and 175% of the mean control 1-kidney filtration (Fig. 5), and hyperperfusion measured by the hippuran clearance showed a similar increase (Fig. 5). Importantly, there were no significant differences in hyperfiltration (on the basis of both creatinine and EDTA clearances) or hyperperfusion between the UNX ♂ NAR and UNX ♀ NAR at any time point after surgery.

Glomerular capillary pressure and blood viscosity

P_{GC} and blood viscosity were measured in a subset of UNX rats 14 weeks after surgery. Proteinuria was not present in any of these rats 11 weeks after UNX, and there was no significant difference in MAP in ♀ and ♂ rats (Table 2). The ♀ NAR were markedly hyperlipidemic as compared to the male NAR. Finally, glomerulosclerosis was not observed in any of these rats. Thus the characteristics of the model described above were also present in this subset. The ♀ rats were considerably smaller than the ♂ rats and left kidney weight, GFR and RPF were lower in UNX ♀ NAR than in UNX ♂ NAR. Stop flow pressure (SFP_0) and colloid osmotic pressure (π) were not significantly different in the UNX ♀ and UNX ♂ NAR, hence P_{GC} was also not significantly different. The free flow pressure in the proximal tubules were also comparable, and therefore there was also no difference in transcapillary glomerular hydrostatic pressure. Plasma viscosity was higher in the UNX ♀ NAR than in UNX ♂ NAR ($P < 0.05$), but there were no differences in whole blood viscosity (Fig. 6).

Plasma apolipoproteins, fibrinogen, viscosity and lipoproteins 37 weeks after UNX

Plasma lipids, apolipoprotein (apo), total protein and fibrinogen concentrations and plasma viscosity in terminal blood samples, 37 weeks after UNX or sham operation, are listed in Table 3. Both plasma apo A-I ($P < 0.01$) and apo E ($P < 0.05$) levels are higher in ♀ than in ♂ rats. UNX increases plasma apo B concentration ($P < 0.01$), and causes a further increase in apo A-I levels ($P < 0.01$) in ♀ rats. UNX had no effect on any of the plasma apolipoproteins in the ♂ rats. The higher total protein levels in the ♀ NAR ($P < 0.01$) are not caused by high fibrinogen levels. On the contrary, fibrinogen levels are slightly higher in the ♂ NAR ($P < 0.05$). Even in the presence of lower fibrinogen, higher plasma lipid and total protein levels in the ♀ NAR caused a significant increase in plasma viscosity ($P < 0.01$).

Lipoprotein cholesterol and triglyceride levels are presented in Figure 7. There were large increases ($P < 0.01$) in the apo

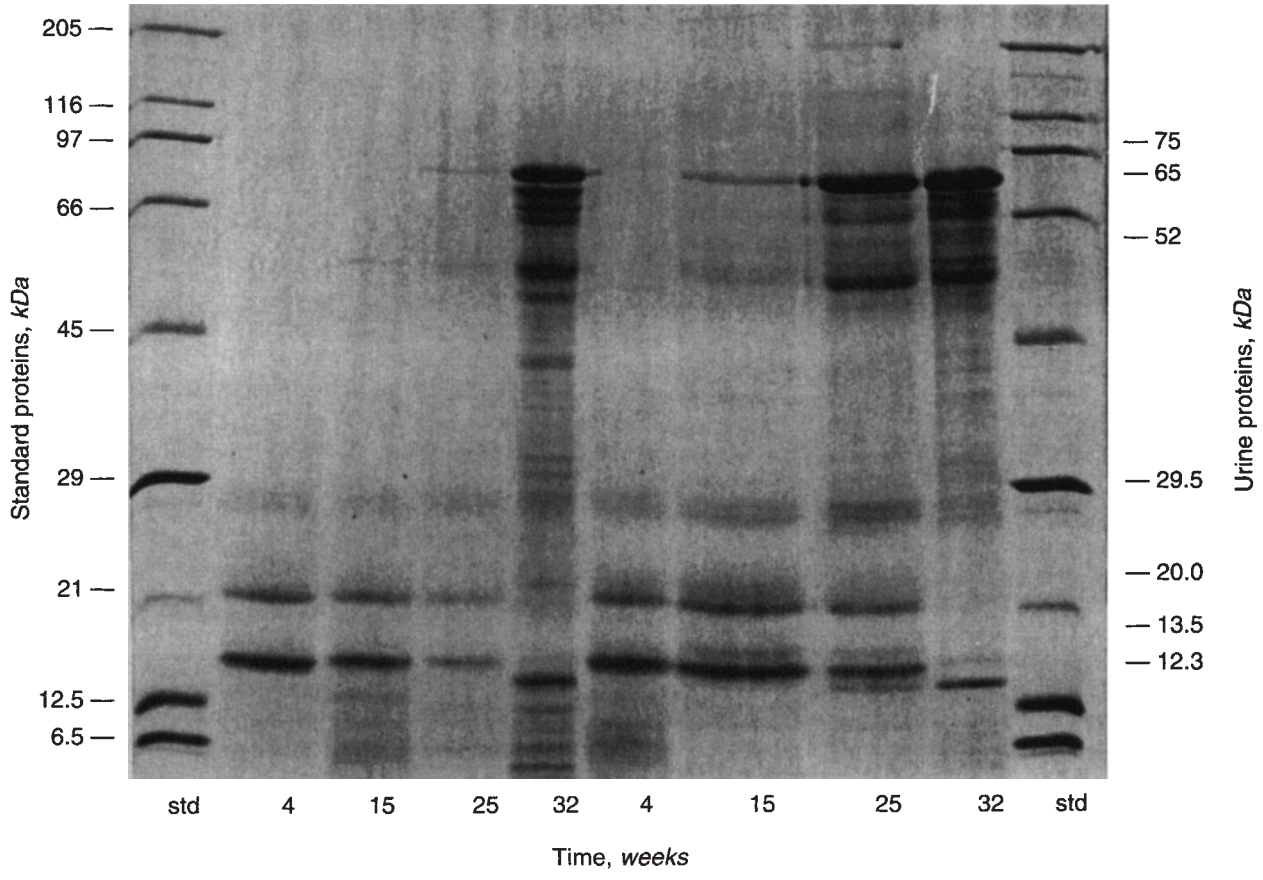

Fig. 4. *SDS-polyacrylamide gel electrophoresis of proteins in consecutive urine samples obtained from 2 female analbuminemic rats at 4, 15, 25 and 32 weeks after uninephrectomy (UNX).* Note that at 25 weeks after UNX one of the rats is already proteinuric. The molecular masses (kDa) of the proteins in the standard mixture are indicated on the left. The approximate molecular masses (kDa) of the major protein bands are indicated on the right.

B-containing lipoproteins IDL and LDL1 as well as in the large HDL-type lipoproteins (LDL2 and HDL2) in the ♀ NAR versus the ♂ NAR. No significant differences were observed in HDL3. UNX as compared to 2K caused a further increase in all lipoproteins except HDL3 in the ♀ NAR ($P < 0.01$). UNX also caused an increase in VLDL cholesterol in the ♂ NAR ($P < 0.05$). There were large increases ($P < 0.01$) in all lipoproteins in the ♀ NAR versus the ♂ NAR. Neither in the ♀ nor in the ♂ NAR was UNX associated with a further increase in triglyceride level in any of the lipoproteins.

Renal apolipoprotein and lipid deposition and glomerular macrophages 37 weeks after UNX

Apo B immunoreactivity was observed in sclerotic lesions of the UNX ♀ rats (Fig. 8A). Glomerular visceral epithelial cells did not reveal apo B staining. Deposition of apo B was not observed in any of the other groups. Weak staining for apo E was found in glomerular visceral epithelial cells and in the mesangium of UNX ♀ rats (Fig. 8B). Mesangial deposition was mainly present in sclerotic areas. In all other groups trace amounts of apo E were observed in visceral epithelial cells, but not in the mesangium. The proximal tubules stained strongly for apo E in all animals (Fig. 8B). Moderate immunostaining was observed for apo A-I in

Table 2. Urinary protein 11 weeks after surgery and plasma lipids, renal hemodynamics and glomerular capillary pressure 14 weeks after surgery in uninephrectomized (UNX) female (♀) and male (♂) analbuminemic rats

	UNX ♀	UNX ♂
N	6	6
Body weight *g*	251 ± 19[b]	475 ± 20
Kidney weight *g*	1.11 ± 0.12[b]	2.13 ± 0.14
Urinary protein *mg/day*	10 ± 2[b]	24 ± 3
Mean arterial pressure *mm Hg*	114 ± 6	118 ± 5
Cholesterol *mmol/liter*	7.5 ± 0.6[a]	5.5 ± 0.3
Triglycerides *mmol/liter*	5.5 ± 0.7[b]	1.8 ± 0.2
Glomerular filtration rate *ml/min*	1.41 ± 0.15[a]	1.91 ± 0.15
Renal plasma flow *ml/min*	3.59 ± 0.47[a]	5.66 ± 0.67
Stop-flow pressure *mm Hg*	46.5 ± 2.9	45.6 ± 1.7
Colloid osmotic pressure *mm Hg*	12.0 ± 0.7	11.1 ± 0.4
Glomerular capillary pressure *mm Hg*	58.5 ± 3.6	56.7 ± 1.9
Proximal tubule pressure *mm Hg*	12.8 ± 0.8	13.2 ± 2.1
Glomerular transcapillary hydraulic pressure *mm Hg*	45.7 ± 2.9	43.5 ± 3.7

Data are mean ± SEM.
[a] $P < 0.05$ vs. ♂ [b] $P < 0.01$ vs. ♂

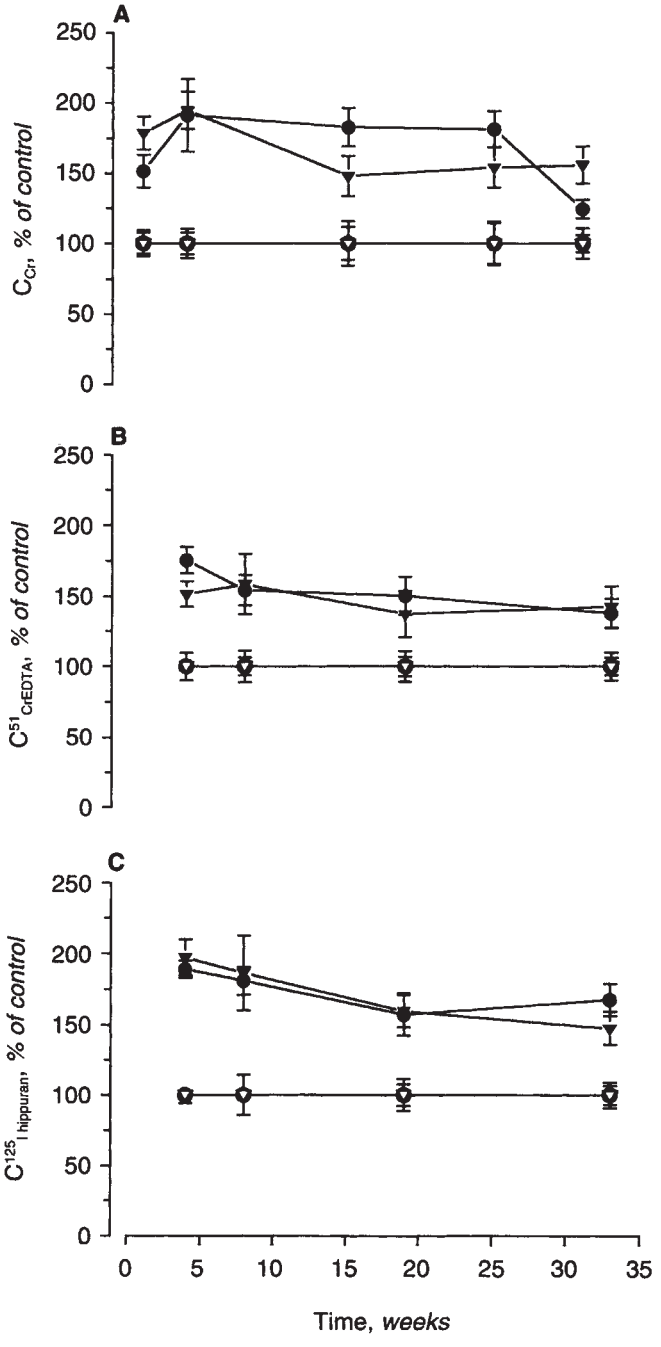

Fig. 5. *Creatinine (A), ^{51}Cr-EDTA (B) and ^{125}I-hippuran (C) clearances in uninephrectomized (UNX) and control (2K) female and male analbuminemic rats.* Symbols are: (○) female 2K; (●) female UNX; (▽) male 2K; (▼) male UNX. Values have been normalized to 50% of the mean value found in control (2K) animals.

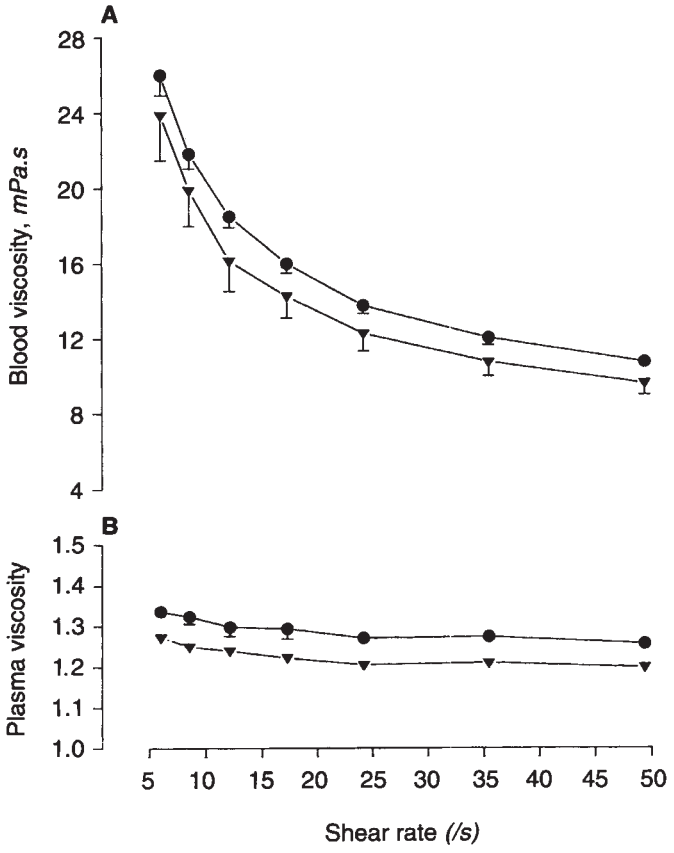

Fig. 6. *Blood (A) and plasma (B) viscosity in uninephrectomized (UNX) female (●) and male (▼) analbuminemic rats.*

Table 3. Plasma cholesterol, triglyceride, apolipoprotein (Apo), total protein and fibrinogen concentrations and plasma viscosity in control (2K) and uninephrectomized (UNX) female (♀) and male (♂) analbuminemic rats 37 weeks after surgery

	2K ♀	UNX ♀	2K ♂	UNX ♂
N	6	10	7	8
Cholesterol *mmol/liter*	11.9 ± 0.6[b]	14.3 ± 0.9[b,c]	6.0 ± 0.3	6.4 ± 0.5
Triglycerides *mmol/liter*	6.9 ± 1.3[b]	6.0 ± 1.3[b]	1.7 ± 0.3	3.0 ± 0.6
Apo A-I *g/liter*	1.58 ± 0.08[b]	2.00 ± 0.12[b,d]	0.83 ± 0.06	0.81 ± 0.07
Apo B *A.U.*	204 ± 8	285 ± 22[b,d]	204 ± 13	218 ± 8
Apo E *g/liter*	0.30 ± 0.01[a]	0.31 ± 0.01[a]	0.24 ± 0.01	0.27 ± 0.01
Total protein *g/liter*	61.7 ± 0.9[b]	63.3 ± 1.0[b]	50.4 ± 1.4	51.1 ± 0.8
Fibrinogen *g/liter*	13.0 ± 1.1[b]	13.7 ± 1.1[a]	17.8 ± 1.3	17.4 ± 0.5
Viscosity *mPa · s*	1.73 ± 0.06[b]	1.89 ± 0.08[b]	1.43 ± 0.04	1.50 ± 0.06

Apo B concentrations are relative to a standard rat plasma pool with a concentration of 100 arbitrary units (A.U.). Data are mean ± SEM.
[a] $P < 0.05$ vs. ♂ [c] $P < 0.05$ vs. 2K
[b] $P < 0.01$ vs. ♂ [d] $P < 0.01$ vs. 2K

glomerular visceral epithelial cells of the UNX ♀ rats (Fig. 8C). In 2K ♀ rats and in both 2K and UNX ♂ rats trace amounts of apo A-I were focally present. Small amounts of apo A-I were found in the glomerular mesangium. In all animals strong immunoreactivity for apo A-I was found at the luminal side of proximal tubular cells in a granular staining pattern, suggestive of lysosomal structures (Fig. 8C). This was not unexpected as the proximal tubule is an important site of apo A-I catabolism [25].

Glomerular lipid deposits were considerably increased ($P <$

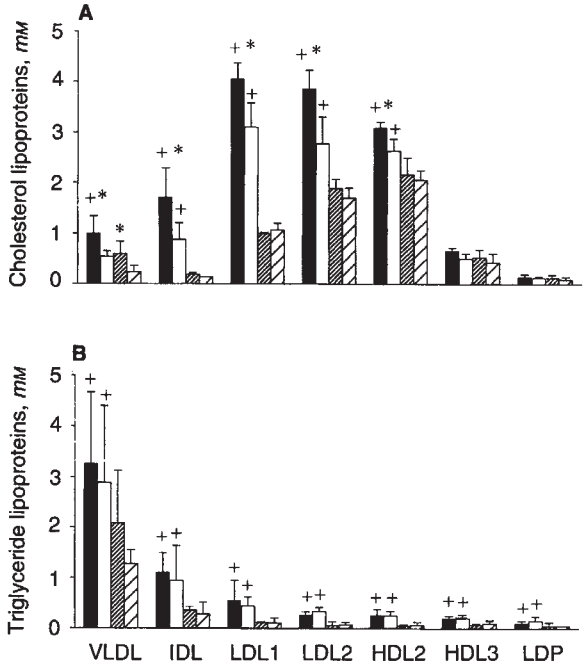

Fig. 7. *Plasma lipoprotein cholesterol (A) and plasma lipoprotein triglycerides (B) in uninephrectomized (UNX) and control (2K) female and male analbuminemic rats.* Symbols are: (■) female UNX; (□) female 2K; ( ) male UNX; (▨) male 2K. Females vs. males $+P < 0.05$; UNX vs. 2K $*P < 0.05$. Data are mean ± SD. Abbreviations are: VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; LDP, lipoprotein deficient plasma.

0.01) in UNX ♀ (score: 89 ± 21), as compared to 2K ♀, UNX ♂ or 2K ♂ (respectively 7 ± 2, 4 ± 2 and 5 ± 2). The median number of macrophages per glomerulus correlated with the glomerular lipid deposit score, being 1.35 in UNX ♀, as compared to 0.32, 0.28 and 0.10 in 2K ♀, UNX ♂ and 2K ♂, respectively. Thus the UNX ♀ rats showed a considerable increase in glomerular apolipoprotein and lipid deposition and glomerular macrophages as compared to the 2K ♀ rats, whereas there was very little glomerular apolipoprotein and lipid deposition and very few glomerular macrophages in the ♂ rats.

Glomerular diameter, glomerulosclerosis and interstitial changes 37 weeks after UNX

Mean glomerular diameter was similar in 2K ♀ and ♂ NAR (respectively 158 ± 2 and 157 ± 4 μm; Fig. 9). UNX increased glomerular diameter in both ♀ and ♂ animals ($P < 0.05$); however, the increase was more pronounced in the ♂ rats ($P < 0.05$).

Glomerulosclerosis was present in 7 of the 10 UNX ♀ (Figs. 8 A and B) with an injury score ranging from 8 to 74. Virtually no sclerotic glomeruli were observed in the other groups. Proteinuric animals demonstrated protein casts in dilated tubules, with characteristic interstitial inflammation and tubular atrophy. Interstitial damage was not observed in non-proteinuric animals. Interstitial lipid deposits were increased ($P < 0.01$) in UNX ♀ (score: 3.2 ± 0.2), as compared to 2K ♀, UNX ♂ or 2K ♂ (respectively 1.7 ± 0.5, 0.7 ± 0.4 and 0.2 ± 0.2).

Discussion

The main finding of the present study is that at similar levels of hyperfiltration and even less glomerular hypertrophy, UNX ♀ NAR develop proteinuria, loss of glomerular permselectivity, glomerulosclerosis and mesangial apolipoprotein and lipid deposition, whereas UNX ♂ NAR do not. In addition, in comparison to UNX ♂ NAR, no significant difference in P_{GC} could be detected in UNX ♀ NAR prior to the development of proteinuria. Our findings suggest that, besides the renal hemodynamic changes and renal hypertrophy induced by uninephrectomy, other factors must play a role in the etiology of renal damage in this model. These factors probably include hyperlipidemia and plasma lipoprotein composition. A similar conclusion was reached in a recent study with a very different model, the 2K Dahl salt-sensitive rat, where a cholesterol synthesis inhibitor considerably attenuated the development of proteinuria and glomerulosclerosis while an angiotensin converting enzyme inhibitor did not [26].

Hyperfiltration in ♂ UNX rats has been studied previously in long-term studies evaluating the effect of varying dietary protein intake on the development of proteinuria and glomerulosclerosis [5, 27]. No reports were found directly comparing hyperfiltration in the first months after uninephrectomy in ♂ and ♀ rats of the same strain. However, comparison of the data from two studies from Baylis's group in which ♂ and ♀ 2K [28] and ♂ and ♀ UNX [7] Munich-Wistar rats were studied revealed no major gender-related differences in the degree of hyperfiltration and hyperperfusion after uninephrectomy. On a diet with a protein content similar to that used in the present study, hyperfiltration in ♂ UNX Wistar rats, measured by the EDTA clearance, was maintained at a similar level (about 150% of the mean control one-kidney value) for 48 weeks [5]. However, these rats developed progressive proteinuria after only 12 weeks [5], in contrast to the present findings in ♂ UNX NAR. This supports our contention that factors other than hyperfiltration play an important role in the development of proteinuria and glomerulosclerosis after uninephrectomy. One such factor may be systemic hypertension [10]. In spontaneously hypertensive rats severe proteinuria and glomerulosclerosis were already present six months after UNX [29]. However, tail cuff pressures were not significantly different between ♀ and ♂ NAR in the present study.

Glomerular hypertrophy has also been considered to be a pathogenic link between a reduction in the number of nephrons and the development of glomerulosclerosis [30–32]. However, in the analbuminemic rat used in the present study the degree of glomerular hypertrophy after uninephrectomy did not predict the severity of glomerulosclerosis. In fact, glomerular hypertrophy was more pronounced in UNX ♂ than in UNX ♀ NAR.

It has been suggested that dietary hypercholesterolemia increases P_{GC} via an effect on plasma and blood viscosity [14]. The increase in blood viscosity may also be an important factor contributing to the progression of renal injury [33]. By varying hematocrit and thus modulating blood viscosity, it has been demonstrated that anemia (hypoviscosity) ameliorates and polycythemia (hyperviscosity) accelerates the development of proteinuria in the remnant kidney model [15]. However, neither whole blood viscosity nor P_{GC} were significantly different between UNX ♀ and UNX ♂ NAR. Plasma viscosity was slightly higher in the ♀ than in the ♂ NAR. This is probably related to differences in the concentrations of plasma proteins other than fibrinogen as well as

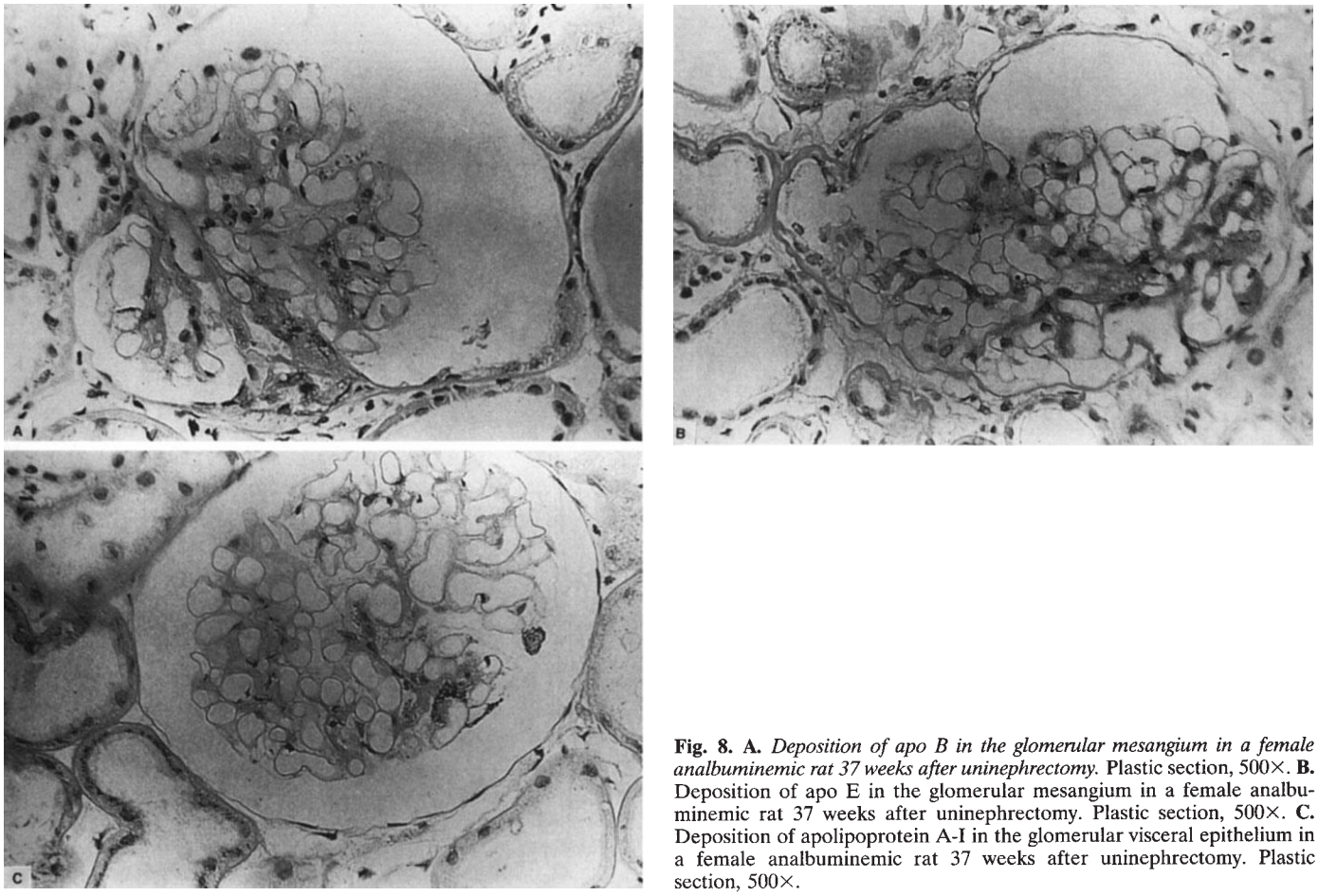

Fig. 8. A. *Deposition of apo B in the glomerular mesangium in a female analbuminemic rat 37 weeks after uninephrectomy.* Plastic section, 500×. **B.** Deposition of apo E in the glomerular mesangium in a female analbuminemic rat 37 weeks after uninephrectomy. Plastic section, 500×. **C.** Deposition of apolipoprotein A-I in the glomerular visceral epithelium in a female analbuminemic rat 37 weeks after uninephrectomy. Plastic section, 500×.

the higher triglyceride levels [34]. Besides severe hyperlipidemia, adult ♀ NAR are characterized by normal total protein levels when compared to normoalbuminemic control rats [23, 35]. This is in contrast to the adult ♂ NAR that are hypoproteinemic [23, 35]. The difference in plasma protein concentration between ♀ and ♂ NAR is due to higher levels of a number of high molecular weight proteins [23], such as transferrin and ceruloplasmin in the ♀ NAR [35]. In this study we demonstrate that plasma fibrinogen, one of the acute phase proteins, is lower rather than higher in ♀ NAR as compared to ♂ NAR. Note that whole blood viscosity was at least an order of magnitude greater than plasma viscosity. These data do not support a substantial role for blood or plasma viscosity and P_{GC} as risk factors for glomerular damage in this model.

The present study suggests that hyperlipidemia and differences in lipoprotein composition may be critical in precipitating the development of proteinuria and glomerulosclerosis after uninephrectomy in the ♀ NAR. Mechanisms postulated to be involved in the nephropathic effects of hyperlipidemia include toxic effects of apo B- and apo E-rich particles, LDL and β-VLDL, respectively, on the glomerular mesangial cell [36–38] and an enhanced influx of macrophages [4, 18]. The ♀ NAR is consistently more hyperlipidemic than the ♂ NAR [12, 39]. This was also the case in the present study where the animals were observed from about two months of age for a period of nearly nine months. The cholesterol and triglyceride content per particle of apo B-containing lipoproteins (Fig. 7) appears to be considerably increased in the ♀ NAR as compared to the ♂ NAR because plasma apo B levels were similar in the two 2K groups (Table 3). This implies that these particles are larger in the ♀ NAR than in the ♂ NAR. Thus a primary difference in lipoprotein composition, that is, not one secondary to proteinuria, may well play a role in the pathogenesis of early glomerular lesions in this model. Indeed, preliminary findings from our department indicate that performing ovariectomy in conjunction with UNX in ♀ NAR profoundly decreases plasma triglycerides and completely prevents the development of proteinuria and glomerulosclerosis [40].

The deposition of lipid and apo B in the glomerular mesangium of the perfusion fixed kidneys was parallel to the increased plasma concentrations of cholesterol and apo B in the proteinuric UNX ♀ NAR. Glomerular deposits of apo B [41, 42] have been observed in biopsies from patients with renal disease, but there was no relation between cholesterol or apo B concentrations and the presence or intensity of immunofluorescence in the glomerulus [42]. Thus it is debatable whether the changes observed in the plasma lipoproteins of subjects that have become proteinuric play an initiating role in the development of renal lesions. Alternatively, they might be considered as being secondary to some other primary pathogenic event, although they may well contribute to

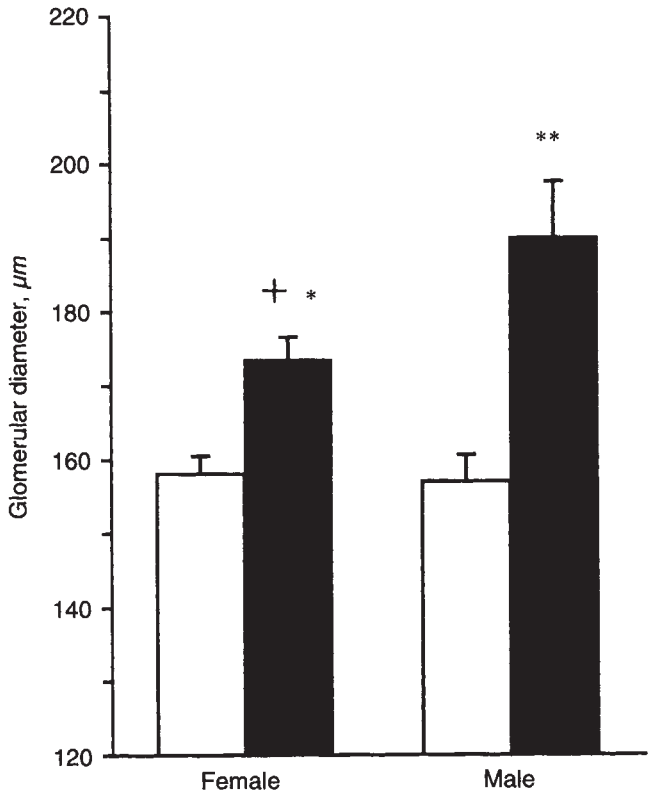

Fig. 9. *Glomerular diameter in uninephrectomized (UNX, ■) and control (2K, □) female and male analbuminemic rats.* Females vs. males $+P < 0.05$; UNX vs. 2K $*P < 0.05$, $**P < 0.01$.

the development of glomerulosclerosis by foam cell formation [4, 43–45].

Plasma apo E levels were higher in the ♀ NAR than in the ♂ NAR, a difference also observed previously in NAR on a conventional diet [12], and this protein species was observed in the mesangium, particularly in sclerotic lesions (Fig. 8B). In another previous study we observed close proximity of apo E to mesangial foam cells in a toxic model of the nephrotic syndrome [44]. Glomerular deposits of apo E [41, 42] have also been observed in biopsies from patients with renal disease. Neither in the present study, nor in a recent report in patients with glomerular injury [42] was a relation observed between plasma apo E levels and incidence or intensity of glomerular immunofluorescence. Therefore, these observations should be interpreted with caution regarding causality in the pathogenesis of the sclerotic lesion. Although macrophages avidly take up apo E-rich particles such as β-VLDL [46], a phenomenon that has also been observed in glomeruli of cholesterol-fed UNX rats [47], it is well known that macrophages synthesize apo E both *in vitro* [48, 49] and *in vivo* when residing in atherosclerotic plaques [50]. Hence both systemic delivery and local production could account for deposition of apo E in the diseased mesangium. In fact, synthesis of apo E by resident macrophages may be the initial step in an autocrine process that draws lipid out of foam cells onto the reverse cholesterol transport pathway [51]. High plasma apo E levels may actually be advantageous. Recently, chronic apo E administration has been shown to reduce the accumulation of cholesterol esters and reduce plaque surface area in Watanabe heritable hyperlipidemic rabbits [52] and transgenic mice overexpressing apo E demonstrate an accelerated clearance of lipoproteins containing apo B [53]. Conversely, severe hypercholesterolemia and atherosclerosis develop in apo E-deficient recombinant mice [54]. Thus both systemic apo E concentrations as well as apo E production by macrophages in the glomerulus may play a role in the regression of glomerulosclerosis.

Plasma apo A-I levels are higher in the ♀ NAR than in the ♂ NAR. Deposition of this protein was mainly localized in the glomerular visceral epithelium, whereas lipid deposition was restricted to the glomerular mesangium. Whether this implies that the small amount of lipid in apo A-I rich particles is below the resolution of the ORO stain or that the apo A-I is circulating as an unbound protein is unclear. Less than 5% of rat plasma apo A-I is unbound [55]; nevertheless, increased traffic of this small fraction through a damaged glomerular basement membrane may account for the positive stain. A similar localization was observed in nephrotic glomeruli from biopsy material [42]. Apo A-I and apo B have been demonstrated in the wall of atherosclerotic vessels: aortae and coronary vessels obtained at autopsy [56] and arteries of renal allografts in chronic rejection [57]. Localization of apo A-I deposition outside the mesangium suggests a subsidiary role in the pathogenesis of glomerulosclerosis.

Summarizing, UNX in ♀ NAR leads to proteinuria after four months and glomerulosclerosis after nine months. Our data indicate that hyperfiltration and glomerular hypertrophy on the one hand and severe hyperlipidemia and altered lipoprotein composition on the other hand play synergistic roles in this model: in the presence of only hyperfiltration and hypertrophy, as in the UNX ♂ NAR, or only hyperlipidemia and altered lipoprotein composition, as in the 2K ♀ NAR, glomerulosclerosis is not found. The relative importance of hyperlipidemia and altered lipoprotein composition in this model can be appreciated by realizing that the presence of additional risk factors in male NAR (higher protein [5, 27] and caloric [58] intake and more rapid body growth [59]), are not as deleterious as the hyperlipidemia and altered lipoprotein composition in female NAR.

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